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Polar and non-polar fraction from *Origanum vulgare* spp. *hirtum* methanolic extract – differences in their bioactivity on *Chlamydomonas reinhardtii* test system

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Abstract

Aim: To compare the bioactivity of both polar and non-polar fraction of *Origanum vulgare* spp. *hirtum* methanolic extract on *Chlamydomonas reinhardtii*.

Material and methods: The polar and non-polar fractions were derived from aerial parts of *Origanum vulgare* ssp. *hirtum*, collected during the flowering stage from the *ex-situ* collection of IBER-BAS. GC/MS analysis of both fractions was done following the standard protocol. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. *Chlamydomonas reinhardtii* 137C+ (WT) was used as a test system. Spot-test, cell survival fraction (SF), test of "visible mutations" and CFGE (for measurement of induced DNA double-strand breaks (DSBs)) were applied.

Results: The polar fraction did not possess genotoxic, mutagenic as well as DNA-damaging effect. The situation with the non-polar fraction was quite different. Even at the lowest concentration of 250 ppm, cell survival was decreased by 60% (SF = 0.41 ± 0.08). Treatment with concentrations equal to/or greater than 500 ppm resulted in around 100% lethality.

A mild mutagenic effect was obtained for the concentration of 250 ppm non-polar fraction (IM = 4.83 ± 0.004). Well-expressed and concentration-dependent induction of DSBs for even the strong DNA fragmentation was observed after the treatment with the non-polar fraction.

Conclusions: The different bioactivity of the two fractions correlated well with their different chemical composition. The polar fraction, rich in sugars, organic acids and flavonoid glycosides, did not possess

genotoxic and mutagenic potential. The strong genotoxic potential of the non-polar fraction might be related to carvacrol content (37.08%), which is not present in the composition of the polar fraction. To the best of our knowledge, this study provides the first information that the carvacrol-rich non-polar fraction of *Origanum vulgare* spp. *hirtum* methanolic extract possesses genotoxic, mutagenic and DNA damaging effect on some low eukaryotes, such as *C. reinhardtii.* Further experiments with carvacrol should be done in order to clarify the exact mechanism of action.

Keywords

Bioactivity, *Chlamydomonas reinhardtii*, polar and non-polar fraction *Origanum vulgare* spp. *hirtum* methanolic extract

Introduction

Origanum vulgare L. (Lamiaceae) is a perennial herb native to the Mediterranean Region and western Eurasia (De Martino et al. 2009; Pezzani et al. 2017). Oregano has been widely applied not only as a flavouring herb, but also for medicinal purposes for centuries. Extensive data exist concerning its antioxidant, antimicrobial, anti-inflammatory and anti-cancer activity (reviewed in Pezzani et al. 2017). Together with these properties, current studies are focused on its promising application as a biopesticide (Ibáñez and Blázquez 2017; Alkan 2020; Grul'ová et al. 2020; Abd-ElGawad et al. 2021). Most of the activities are contributed to the phenolic monoterpenes, thymol and carvacrol (De Santis et al. 2019). Our previous data already revealed that the methanolic extract with a quantity of carvacrol 15.67% is less toxic/genotoxic than the essential oil containing 74.34% carvacrol (unpublished data). Taking into account these data, our present hypothesis is that carvacrol-rich extract fractions would be more genotoxic than those without carvacrol.

The aim of the present work was to compare the bioactivity of polar and non-polar fractions of oregano methanolic extract on *Chlamydomonas reinhardtii*.

Materials and methods

Plant material and extraction procedure

Crude methanolic extract from aerial parts of *Origanum vulgare ssp. hirtum*, collected during the flowering stage from the *ex-situ* collection of IBER-BAS, was obtained by air-drying, extraction by classical maceration with methanol for 24 h, filtration and evaporation to dryness. Liquid/liquid extraction, using distilled water and chloroform, was used in order to separate the non-polar and polar compounds into two fractions.

GC/MS analysis

A total of 50 mg of each fraction was dissolved in 50 µl of pyridine. Then, 50 µl of N,O-bis-(trimethylsilyl)trifluoroacetamide were added and the samples were heated at

70 °C for 2 h. After cooling, the samples were diluted with 300 µl of chloroform and analysed using GC-MS. The GC–MS spectra were recorded on a Thermo Scientific Focus GC, coupled with a Thermo Scientific DSQ mass detector operating in EI mode at 70 eV. The chromatographic conditions have been described by Berkov et al. (2021). The metabolites were identified as TMSi derivatives by comparing their mass spectra and retention indices (RI) with online available plant-specific database. The measured mass spectra were deconvoluted by the Automated Mass Spectral Deconvolution and Identification System (AMDIS), before comparison with the databases. RI of the compounds were recorded with standard n-hydrocarbon calibration mixture (C9- C36) (Restek, Cat no. 31614, supplied by Teknokroma, Spain) using AMDIS 3.6 software.

Toxicity/Genotoxicity

The *C. reinhardtii* strain 137C (+) WT cell suspensions (1×10^6 cells/ml) at the end of the exponential and the beginning of the stationary phase were treated for 10 seconds with six concentrations of polar and non-polar fractions (250, 500, 750, 1000, 3000 and 5000 ppm). The concentrations and exposure time were the same as those previously defined for methanolic extract. Sager-Granick liquid medium (SG) and DMSO 1000 ppm were used as negative controls. As a positive control, 500 ppm Nurelle D was used following commercial recommendations for pest control. In order to calculate the survival fraction of cells (SF) (Bryant 1968), the "clonal" assay, based on colony-forming ability, was performed.

Mutagenicity

The mutagenic potential of polar and non-polar fractions was revealed by the test of "visible mutations". Changes in the size, morphology and pigmentation of surviving colonies were analysed (Shevchenko 1979). The calculations of percentage-induced mutant colonies and index of mutagenicity (IM) have been described in detail previously (Dimitrova et al. 2007).

Test for double-strand breaks (DSBs) induction in DNA

Constant Field Gel Electrophoresis (CFGE) procedure and its advantages for the evaluation of potential DNA damaging capacity of the fractions were described earlier by Chankova and Bryant (2002) and Chankova et al. (2005). The fraction of DNA released (FDR) from the wells was calculated according to the formula presented by Chankova et al (2009) and Dimova et al. (2009).

Data analysis

All data are mean values from at least three independent experiments. GraphPad Prism programme version 6.04 software (San Diego, USA) and one-way analysis of variance ANOVA were used.

Results

GC/MS analysis of polar and non-polar fraction of methanolic extract

Thirty-four compounds were identified in the studied fractions of *O. vulgare* ssp *hirtum* methanolic extract (Table 1). Mono-, di-, trisaccharides, flavonoids, organic and phenolic acids and sugar alcohols were found in the polar fraction. Sucrose (**25**) and 6-hydrohyflavon glycoside (**24**) were determined as main constituents. Monoterpenoids, fatty and triterpene acids, sterols and flavonoid aglycones were identified in the non-polar fraction with the main compound carvacrol (**3**) (37.08%).

Quantities are relative to the percentage of the area of all chromatogram peaks.

Toxicity/Genotoxicity of polar and non-polar fractions

As a first step, the toxic/genotoxic potential of the polar and non-polar fractions was compared using the 'spot' test and survival fraction (SF) assay.

Significant differences were obtained between polar and non-polar fractions with regard to these two endpoints. Spot test data illustrated no concentration-dependent effect of polar fraction – the spot's intensity was identical in all samples, including control samples. These results are informative for the absence of toxic/genotoxic capacity of the polar fraction in this concentrations range (Fig. 1). Quite different was the second picture when non-polar fraction was used – single colonies instead of a spot were formed after the treatment with the lowest tested concentration of 250 ppm and no survived colonies or spots formation were seen after the application of concentrations equal to/or greater than 500 ppm. These results could be attributed to the very strong toxic/genotoxic capacity of the non-polar fraction inducing cell death (Fig. 1, second row).

Our SF results corresponded well with the spot test information. None of the tested polar fraction concentrations decreased the cell survival. The results were comparable with those calculated for the control samples (Fig. 2). On the other side, well expressed toxic/genotoxic potential was found for the non-polar fraction. Even at the lowest concentration of 250 ppm, cell survival was decreased by 60% (Fig. 2). Treatment with concentrations equal to/or greater than 500 ppm resulted in around 100% lethality.

Mutagenicity

Further, the mutagenic potential was evaluated. Again, treatment with any of the tested polar fraction concentrations did not result in statistically significant induction of mutant colonies. Contrarily, 250 ppm of the non-polar fraction was found to possess a mild mutagenic effect (IM = 4.83 ± 0.004) (Table 2). Two types of "visible mutations" in *C. reinhardtii* strain 137C were obtained – low-size and pigmental, which are considered as a result of delayed cell division and point mutations in nuclear or chloroplast

Table 1. Metabolites in the polar and non-polar fraction of *O. vulgare* ssp *hirtum* methanolic extract identified by GC/MS.

Compounds	Retention time (RT)	Retention index (RI)	Polar fraction [%]	Non-polar fraction [%]
Glycerol (**1**)	4.48	1258	3.86	
Succinic acid (**2**)	4.90	1305	0.20	
Carvacrol (**3**)	5.18	1339		**37.08**
Fatty alcohol (**4**)	5.70	1389		1.65
Caryophyllene (**5**)	6.14	1492		1.95
Meso erythrol (**6**)	6.55	1496	0.64	
Pyroglytamic acid (**7**)	7.26	1512	0.6	
Caryophyllene oxide (**8**)	8.15	1517		2.33
4-Hydroxybenzoic acid (**9**)	8.45	1625	0.5	
Fatty acid (**10**)	8.50	1708		1.05
Arabitol (**11**)	9.65	1721	0.16	
Fructose 1 (**12**)	10.65	1800	0.6	
Fructose 2 (**13**)	11.24	1837	4.49	
Fructose 3 (**14**)	11.97	1855	1.66	
Glucose (**15**)	12.59	1882	7.14	
Monosaccharide 1 (**16**)	13.36	1918	0.57	
Monosaccharide 2 (**17**)	14.14	1967	5.47	
Hexadecanoic acid (**18**)	15.48	2041		1.99
Myo-inositol (**19**)	16.04	2080	6.22	
Caffeic acid (**20**)	17.02	2131	0.58	
Octadecatrienoic acid (**21**)	18.46	2211		3.74
Octadecanoic acid (**22**)	21.84	2238		0.56
Disaccharide 1(**23**)	23.17	2501	1.92	
6-OH flavone glycoside (**24**)	24.89	2603	**10.07**	
Sucrose (**25**)	25.18	2628	**26.28**	
Naringenin (flavanone) (**26**)	27.95	2778		0.76
Disaccharide 2 (**27**)	29.60	3202	2.66	
9-Octadecenoic acid (**28**)	29.83	3238		7.85
Taxifolin (flavanonols) (**29**)	30.00	3377	1.14	
ß-Sitosterol (**30**)	36.41	3389		1.69
Disaccharide 3 (**31**)	38.01	3618	1.5	
Rosmarinic acid (**32**)	38.69	3642	0.23	
Triterpene acid 1 (**33**)	42.82	3739		0.84
Triterpene acid 2 (**34**)	44.81	3786		1.61

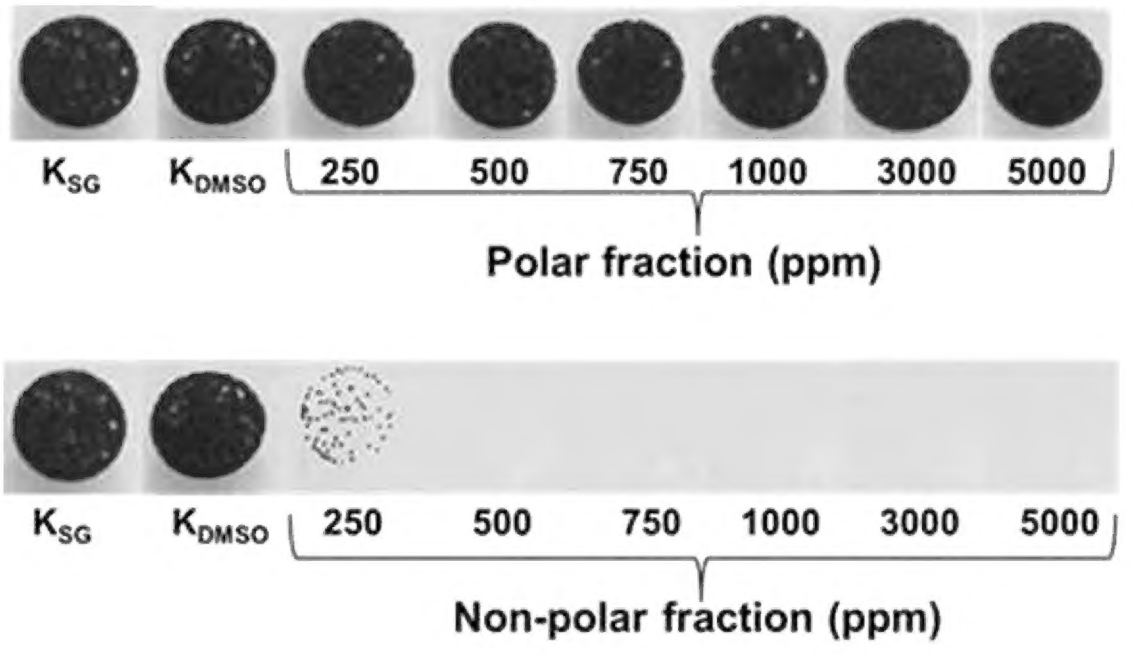

Figure 1. Spot test of *C. reinhardtii* 137C+ after treatment with different concentrations of polar and non-polar fractions of *O. vulgare* ssp *hirtum* methanolic extract.

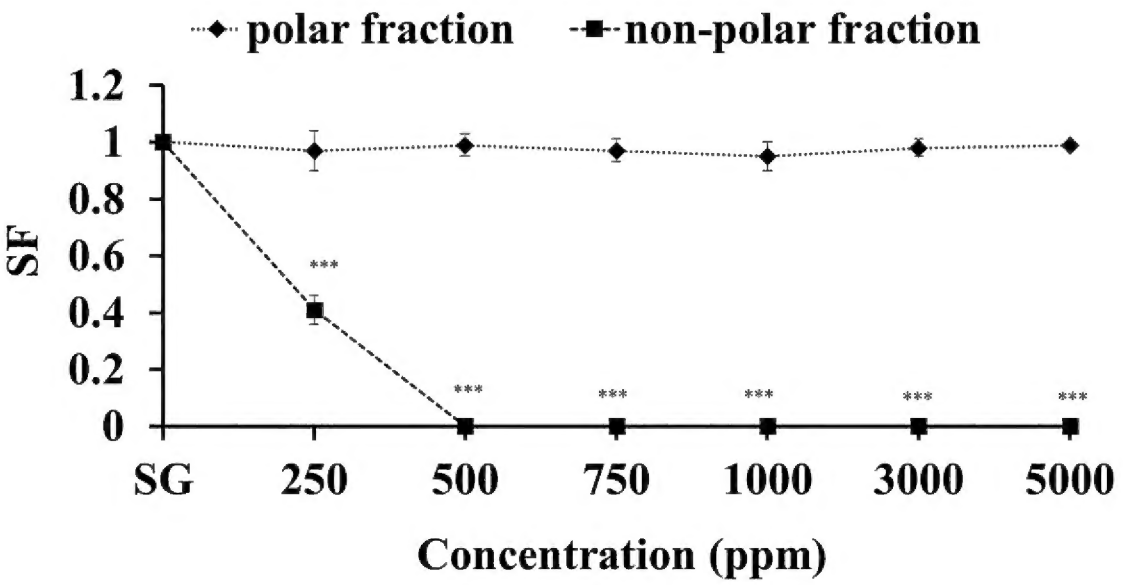

Figure 2. Cells survival fractions (*SF*) after the treatment with polar and non-polar fractions from *Origanum vulgare* methanolic extract. Mean data are from three independent experiments. Error bars represent standard errors of mean values. Where no error bars are evident, errors were equal to or less than the symbols. One-way ANOVA analysis reveals statistically significant differences between SG vs. all concentrations non-polar fraction (***P < 0.0001).

Table 2. Mutagenic activity of the polar and non-polar fraction of *Origanum vulgare* spp. *hirtum* methanolic extract in *C. reinhardtii* 137C. One-way ANOVA analysis reveals statistically significant differences between SG vs. all concentrations non-polar fraction (ns: non-significant; ***P < 0.0001).

Variants	SF	"Visible" mutations (%)	Mutagenic index (IM)
SG	1	0.098 ± 0.0002	
DMSO 1000 ppm	0.99 ± 0.001^{ns}	0.117 ± 0.0002 ^{ns}	0.198
250 ppm polar fraction	0.97 ± 0.07 ^{ns}	0.120 ± 0.0003 ^{ns}	0.232
500 ppm polar fraction	0.99± 0.04 ^{ns}	0.110 ± 0.0002 ^{ns}	0.128
750 ppm polar fraction	0.97 ± 0.04 ^{ns}	0.119 ± 0.0005 ^{ns}	0.227
1000 ppm polar fraction	0.95 ± 0.05 ^{ns}	0.114 ± 0.0003 ^{ns}	0.169
2000 ppm polar fraction	0.98 ± 0.03 ^{ns}	0.131 ± 0.0005 ^{ns}	0.358
3000 ppm polar fraction	0.99 ± 0.002 ^{ns}	0.118 ± 0.0003 ^{ns}	0.210
250 ppm non-polar fraction	0.41 ± 0.08***	0.569 ± 0.001***	4.83

DNA, respectively. The absence of "visible mutations" after the treatment with a non-polar fraction in the concentration range of 500–5000 ppm could be explained with the 100% cell lethality.

DNA damaging potential

Our findings illustrated no DNA damaging capacity of the polar fraction – no statistically significant higher levels of DSBs were calculated after the application of the tested concentrations (Fig. 3). Well-expressed and concentration-dependent induction of DSBs for even the strong DNA fragmentation was observed after the treatment with a non-polar fraction (Fig 3). No statistically significant difference between DSBs levels measured after the treatment with 750 and 1000 ppm of the non-polar fraction was scored (p > 0.05).

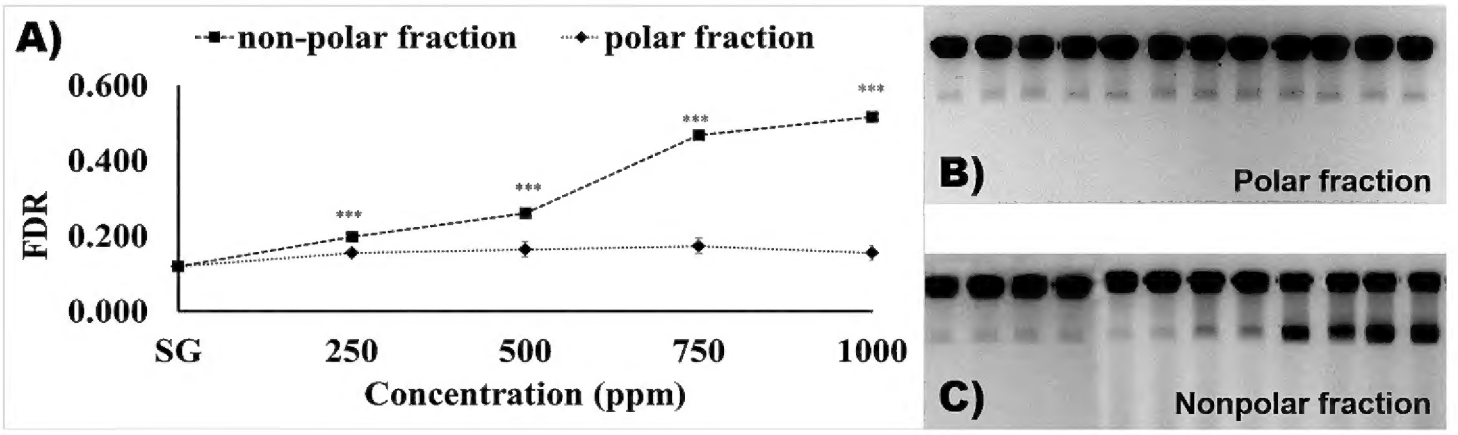

Figure 3. DSBs induced after the treatment with polar and non-polar fractions of *Origanum vulgare* spp. *hirtum* methanolic extract. Mean data are from three independent experiments. Error bars represent standard errors of mean values. Where no error bars are evident, errors are equal or less than the symbols. One-way ANOVA analysis reveals statistically significant differences between SG vs. all concentrations of the non-polar fraction (***$P < 0.0001$).

Discussion

The present work provides evidence for the differences in the bioactivity of polar and non-polar fractions isolated from *Origanum vulgare* spp. *hirtum* methanolic extract.

The polar fraction was shown to possess no toxic/genotoxic, mutagenic or DNA damaging potential on *Chlamydomonas reinhardtii* WT 137C.

Contrary to these results, the well-expressed genotoxic, mutagenic and DNA damaging potential of the non-polar fraction was revealed, depending on the concentration applied. SF data revealed a very strong decrease of cell survival in the range of 250 to 500 ppm, after that no effect of concentration was found. No survived colonies after the treatment with concentrations in the range 500–1000 ppm were scored. A concentration-dependent increasing of non-polar fraction induced DSB was found up to 750 ppm; after that, statistically not significant differences were calculated. Comparing the concentration-effect curves for the evaluation of SF and DSBs induced, we found that they are not completely similar. This confirmed our previous finding with Zeocin that other mechanisms in addition to DSB induction probably could be involved in the formation of cell death (Chankova et al. 2007).

Significant differences in the phytochemical content were obtained. The polar fraction was found rich in sucrose, while the main constituent of the non-polar one was carvacrol. Based on the reported results, it could be suggested that the strong genotoxic effect of the non-polar fraction could be attributed to the presence of carvacrol. Additionally, such difference could be explained with the previously reported data that essential oils and non-polar extracts are better absorbed into the algae cells than methanolic and polar extracts (Yi et al. 2011). Lipophilic compounds, such as carvacrol, may induce alterations to the cell membrane physico-chemical properties and thus allowing easier entry into the cells (Ben Arfa et al. 2006; Yi et al. 2011; Barani et al. 2015).

Based on this, it could be suggested that the carvacrol as a main constituent of the fraction could be responsible for the toxic/genotoxic and DNA damaging potential,

inducing DSB. At present, the available data in the literature reported the potential phytotoxicity of carvacrol, based mainly on results concerning seed germination, root length etc. (Kordali et al. 2008; Koiou et al. 2020; Zhou et al. 2021). De Assis Alves et al. (2018) provided evidence for the genotoxic properties of carvacrol on *Lactuca sativa* and *Sorghum bicolor*. Carvacrol has been classified with chronic aquatic toxicity according to CLP (Directive 1272/2008/EC) and European Chemicals Agency (ECHA) (https://echa.europa.eu/bg/registration-dossier/-/registered-dossier/23562/6/1).

Our study provides new data that the non-polar fraction at a concentration of 250 ppm possesses mild mutagenic activity by induction of mutations related to delayed cell division and point mutations in nuclear or chloroplast DNA. These results are in accordance with another study where carvacrol is reported to affect the plant cell cycle (Pinheiro et al. 2015). Additionally, this fraction is found to induce double-strand breaks in DNA. All of this provides evidence for the genotoxic potential of fractions with a high quantity of carvacrol.

Conclusion

Based on the reported data, it could be concluded that the different bioactivity of the two fractions correlates well with their different chemical composition. The polar fraction, rich in sugars, organic acids and flavonoid glycosides, did not possess genotoxic, DNA damaging and mutagenic potential.

To our present state of knowledge, this study provides the first information that the carvacrol-rich (37.08%) non-polar fraction of *Origanum vulgare* spp. *hirtum* methanolic extract possesses a genotoxic, mutagenic and DNA-damaging effect on some low eukaryotes, such as *C. reinhardtii*. Further experiments with carvacrol should be done in order to clarify the exact mechanism of action.

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